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Pleiotrophic Functions of Epstein-Barr Virus Nuclear Antigen-1 (EBNA-1) and oriP Differentially Contribute to the Efficacy of Transfection / expression of Exogenous Gene in Mammalian Cells

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Abstract

The EBNA1 gene and oriP sequence, originally derived from the EBV genome, provide plasmid vectors with artificial chromosome (AC)-like characteristics, including cytoplasm-to-nuclear transport, nuclear retention, replication and segregation of the DNA, while transcriptional up-regulation has been suggested as another activity of the EBNA1/oriP. Transfection as well as expression rates of various nonviral delivery vehicles are highly improved by inserting these genetic elements into plasmid DNA constructs. Here we differentially analyzed the contribution of each function of the EBNA1/oriP to the efficacy of electroporation-mediated genetic delivery and expression in mammalian cells. It was found that the EBNA1/oriP-mediated acceleration of genetic delivery and expression was predominantly due to the promotion of cytoplasm-to-nuclear recruitment as well as enhancement of transcription, while the episomal replication of the EBV-AC was not essentially involved.

1. Introduction

To analyze and manipulate the mammalian cell functions, efficient and high-throughput technologies to transfer genetic information into cells and tissues are required. However, the critical impediment is that the efficacies of delivery and transgene expression have been limited, as far as conventional nonviral delivery systems are used.

The EBNA1 gene and oriP are important genetic elements of the Epstein-Barr virus (EBV). EBNA1 is a nuclear phosphoprotein with sequence-specific DNA binding activity, while its consensus binding motifs are repetitively present in the dyad symmetry (DS) and the family of repeats (FR) elements in the oriP (reviewed in (Mazda, 2000; Tsurumi et al., 2005)). EBNA1 is the only viral protein necessary and sufficient to induce replication and maintenance of the EBV genome in the cells latently infected with the virus. Insertion of EBNA1 gene and oriP sequence into recombinant plasmid DNA provides the constructs with artificial chromosome (AC)-like characteristics (reviewed in (Mazda, 2002; Mazda and Kishida, 2005)). We previously showed that the transfection/expression efficiency of nonviral delivery systems is also highly improved by using EBV-based AC, when they were transfected into cultured cells by means of the cationic polymer, cationic liposome or electroporation (reviewed in (Mazda, 2000; Mazda and Kishida, 2005)). The EBV-AC also enables quite efficient genetic transduction *in vivo* into a variety of organs and tumors in animals, using synthetic compounds, cationic emulsion, electroporation, gene gun, sonoporation and naked DNA procedures (reviewed in (Mazda, 2002; Mazda and Kishida, 2005)).

The mechanisms underlying the efficient transfection/expression have not been fully understood, however, because the EBNA1 exerts a variety of functions through sequence-specific binding to oriP. Although EBNA1 was originally shown to support DNA replication in latently infected human cells (Lupton and Levine, 1985; Rawlins et al., 1985; Reisman et al., 1985; Yates et al., 1985), it was also demonstrated that the EBNA1 promotes cytoplasm-to-nuclear entry of the oriP-bearing plasmid

(Ambinder et al., 1991; Fischer et al., 1997), retention of the plasmid in the nucleus (Krysan et al., 1989; Jankelevich et al., 1992; Middleton and Sugden, 1994), and transcription (Reisman and Sugden, 1986; Sugden and Warren, 1989; Wysokenski and Yates, 1989; Gahn and Sugden, 1995; Puglielli et al., 1996). These functions may partly contribute to the efficient transfection and expression of the EBV-based plasmid vectors, but the contribution of each function remains to be clarified.

In the present study we investigated the mechanisms underlying the high-level gene transfer and expression achieved by the EBNA1/oriP. The results may provide fundamental rationale of the mechanisms of the EBV systems in genomic manipulation of cells and tissues. Moreover, the results may also offer important information to devise and improve nonviral gene manipulation systems.

2. Material and method

2.1 Plasmids.

The pGVP (Fig. 1, upper left) contains the Luc gene downstream of the SV40 minimal promoter. DNA fragments corresponding to oriP (AccI-BamHI 2.5 kb), FR (HaeIII-HaeIII 0.86 kb) and DS (EcoRV-ApaI 0.25 kb) were inserted into the pGVP to generate pGVP-oriP, pGVP-FR and pGVP-DS, respectively (Fig. 1, upper left). Other plasmids were previously described (Fig. 1) (Tomiyasu et al., 1998; Cui et al., 2001; Kishida et al., 2001). Plasmids were purified using Qiagen MaxiPrep Endo-free kits (Qiagen, Hilden, Germany).

2.2 Cells

HeLa (a human cervical carcinoma cell line), NIH3T3 (a murine fibroblast cell line), CT-26 (a murine colon carcinoma cell line), K562 (a human erythroleukemia cell line) and B16 (a murine melanoma cell

line) were maintained under standard conditions. Transfectants constitutively expressing EBNA1 (HLE, CT-26E, 3T3E and B16E) were established from HeLa, CT-26, NIH3T3 and B16 cells, respectively, as previously described (Mazda et al., 1994; Mazda et al., 1997). Luc gene stable transfectants were established by co-transfecting HeLa, CT-26 and NIH3T3 cells with linearized pGVP-oriP and pHyg (a plasmid encoding Hygromycin resistant gene) followed by Hygromycin selection (HeLa/oriP-Luc, CT-16/oriP-Luc and 3T3/oriP-Luc cells, respectively). For cell-cycle synchronization, cells were cultured in FBS-free medium for 1 day. After washing, the culture supernatant was replaced by fresh medium supplemented with 10% FBS and 5 mg/ml of aphidicholine, followed by culturing for another 1 day.

2.3 Transfection and reporter assays

Electroporation was performed as described (Mazda et al., 1997). Luc and β-gal assays were performed as described elsewhere (Kishida et al., 2001; Nakanishi et al., 2003).

2.4 Southern blot and real-time PCR analyses of cytoplasmic and nuclear DNA

Cell were washed five time with PBS(-) and resuspended in 0.5 ml of lysis byffer (0.5% Nonidet P40, 10 mM NaCl, 3 mM $MgCl_2$, 10 mM Tris-HCl pH7.4) to dissolve the plasma membrane. After centrifugation at 1,400 x *g*, the supernatant was collected (cytoplasmic fraction). The procedure was repeated twice and the final pellet was incubated with 0.1 mg/ml Proteinase K (Nacalai Tesque) for digestion of nuclear membrane and used as the nuclear fraction. DNA was extracted from the cytoplasmic and nuclear fractions using genomic DNA purification kit (TOYOBO, Osaka, Japan). Southern blot analysis was performed by a standard method, using 2.5 kb XhoI fragment derived from pGVP-oriP as a probe. Real-time PCR was performed using the 7300 Real Time PCR System (Applied Biosystems, Foster city, CA, USA). The matching primers and dye probe for the oriP sequence (Forward Primer: 5'-GTGAGATGGACATCCAGTCTTTACG; Reverse Primer: 5'-GGGCAATAAATACTAGTGTAGGAATGAAACATT;

Reporter: 5′-CCCACCCCATGGATTT) was purchased from Applied Biosystems (Applied Biosystems).

3. RESULTS

3.1 Roles of EBNA1, FR and DS in high rate transfection and expression achieved by EBV-AC.

We first examined the expression levels of Luc marker gene in cells transfected with EBV-based AC or conventional plasmid vectors. Transfection with pGEG.Luc resulted in approximately 7-times (day 2) and 30-times (day 4) higher expression of the marker gene in B16 cells compared with the transfection with pG.Luc (Fig. 2). The data are consistent with our previous results demonstrating the drastic high efficacy of the EBV-AC in electro-transfection into cultured cells (Mazda, 2002; Mazda and Kishida, 2005).

To differentially assess the functions of EBNA1 and oriP, we conducted co-transfection experiments. DNA fragments corresponding to whole oriP, DS, or FR were inserted into pGVP reporter plasmid, and co-transfected with pG.β (as an internal control) into stable transformants that had been engineered to express EBNA1 constitutively (HLE, 3T3E and CT-26E). As shown in Fig. 3 left panels, EBNA1-negative cells expressed the Luc at quite low levels regardless of the oriP-derived sequences. In striking contrast, EBNA1-positive cells exhibited tremendously strong expression of Luc, provided that the the reporter construct contained full length oriP.

3.2 Activation of transcription by EBNA1/oriP.

HeLa/oriP-Luc, CT-16/oriP-Luc and 3T3/oriP-Luc cells possessed in their chromosome the oriP and the Luc gene under the control of SV40 minimum promoter. Super-transfection of pOS.E into these cells resulted in significant augmentation in the Luc expression (Fig. 4). This augmentation should be due to transcriptional enhancement, rather than the EBNA1-mediated promotion of extracellular-to-intranuclear

transfer or plasmid replication. The EBNA1 indispensably contributed to this phenomenon, as demonstrated by the impairment of Luc expression by a frame-shift mutation within the EBNA1 coding sequence (Fig. 4). Consistent results were obtained using three independent transformant cell lines for each construct.

3.3 DNA replication does not essentially contribute to the high rate transfection/expression of the EBV-AC.

To assess possible roles of DNA replication in the high rate transfection/expression of EBV-AC, HLE, 3T3E and CT-26E cells were transfected with pGVP-oriP, and extrachromosomal DNA was subjected to the replication assay. As results, replication of oriP sequence was demonstrated only in human cells (data not shown), providing evidence that the robust transgene expression in the murine cells (Figs. 2-4) was independent of the episomal replication.

3.4 Cytoplasm-to-nuclear transportation of oriP-bearing plasmid DNA.

The elevation in transfection/expression efficacies may, at least partly, be attributed to EBNA1-mediated transfer of oriP-bearing plasmids into the nucleus. To assess this possibility, nucleus and cytoplasm were separated from pGVP-oriP-transfectants and amount of oriP DNA was estimated by Southern blotting. As shown in Fig. 5A, the plasmid DNA was detected in the nuclei of EBNA1-positive cells 8 h after the transfection, while EBNA1-negative cells possessed the oriP-containing plasmid exclusively in the cytoplasm. In these experimental settings, the cytoplasm-to-nuclear translocation of the plasmid may not be resulted from nuclear membrane breakdown at the M phase of the cell-cycle, because the cell-cycle had been synchronized at G0/G1 before the transfection (Fig. 5B). Similar results were also obtained by quantitive real-time PCR analysis of cell fractions of EBNA1-positive HLE or EBNA1-negative HeLa cells transfected with pGVP-oriP (Fig. 6A).

Then we examined whether this phenomenon is also seen in murine cells. As shown in Fig. 6B and C, neither CT26 nor B16 possessed oriP-bearing DNA in the nucleus at a detectable level, whereas it was significantly demonstrated in the nucleus of EBNA1-expressing transformants, albeit at smaller copy numbers than those detected in cytoplasm.

4. Discussion

The genetic transfer and expression by nonviral delivery systems require several steps, including the passage of plasmid DNA through plasma membrane, entry of plasmid DNA into nucleus, and transcription. To develop efficient nonviral delivery systems for cultured cells and organs, the efficacy of each step should be independently analyzed and appropriately improved. It sometimes tends to be considered that the efficiency of "transfection" mostly depend on the efficiency of DNA transport from extracellular space to intracellular space, and this is one of the reasons why a variety of nonviral gene delivery vehicles (cationic lipids, cationic polymers, ultrasound, electrotransfer, etc.) are being developed and modified in an attempt to improve the efficiency of "transfection" and transgene expression. Obviously the rate of extracellular-to-intracellular transport is an important step that is capable of controlling the efficiency of transfection and expression. However, some studies suggested that the DNA transportation from the cytoplasm to the nucleus is the most critical step that determine the efficiency of transfection and expression (Lechardeur and Lukacs, 2002; Hebert, 2003), which is also consistent with our present results. We also suggested that the extracellular-to-intracellular transport of exogenous DNA has already reached a satisfactory level, in case some delivery systems such as electroporation are used, because transfection with EBV-AC results in robust expression of transgene in nearly 100% of cells under the optimized conditions (Mazda and Kishida, 2005).

Although plasmid vectors containing the EBNA1 gene and oriP efficiently replicates in cultured

cells of human origin (Yates et al., 1985), accumulating evidence implies that the plasmid is not capable of replicating in rodent cells (Yates et al., 1985; Krysan and Calos, 1993). Intriguingly, however, we have shown that the EBV-AC enables enormous expression of transgene not only in human but also in rodent cells (Tomiyasu et al., 1998; Nakanishi et al., 2003; Asada et al., 2002). In the meanwhile, regardless of the species, the robust transgene expression can be apparently elicited as early as the next day of the gene transfer without any drug selection (Tomiyasu et al., 1998; Asada et al., 2002; Nakanishi et al., 2003). These findings suggest that the EBNA1-mediated replication of oriP-bearing DNA may not contribute to the high efficiencies of transfection/expression achieved by EBNA1/oriP.

Another important factor that may affect the efficiencies of transfection/expression of EBV-AC is the function of oriP as transcriptional enhancer that is facilitated by the EBNA1. This function has been suggested by several previous reports (Reisman and Sugden, 1986; Sugden and Warren, 1989; Wysokenski and Yates, 1989; Gahn and Sugden, 1995; Puglielli et al., 1996). By the conventional transient transfection assay systems, however, it is not easy to discriminate between the contribution of cytoplasm-to-nuclear transport and that of transcriptional enhancement to the efficacy of transgene expression. Indeed, the efficiency of cytoplasm-to-nuclear transport most heavily influences the transient expression rate of the marker gene as suggested by Figs. 3 and 4. Nevertheless, the present findings indicated that, in both human and murine cells, the EBNA1 was capable of trans-activating the transcription of the marker gene that located *in cis* to the oriP, even without involvement of intranuclear transport of plasmid DNA by EBNA1 (Fig. 4).

It has not been fully unveiled in which intracellular compartment the EBNA1 and exogenous oriP-bearing plasmid interact to each other after transfection.

EBNA1 may interact and cooperate with some host molecule(s) to exert its functions. ORC and MCM were shown to be the cofactors that support replication of oriP-bearing DNA by EBNA1 (Chaudhuri et al., 2001; Dhar et al., 2001; Hirai and Shirakata, 2001). On the other hand, it has not been fully

elucidated whether other functions of EBNA1 are also restricted to primate cells, although we previously showed that at least some EBNA1/oriP function(s) should be exerted in murine and rat cells (Tomiyasu et al., 1998). The present findings strongly suggest that both cytoplasm-to-nuclear transfer and transcriptional enhancement were active in rodent cells. As for the cofactor(s) required for the cytoplasm-to-nuclear transfer and that (those) for transcriptional up-regulation, rodent factors may be substitutable for human ones, probably because these molecules are highly conserved in mammalians, although further molecular analyses are required to unveil this enigmatic species specificity issue of the EBNA1/oriP system.

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Legends to the Figures

FIG. 1. Schematic diagrams of the plasmid vectors. Constructions of pGVP with/without oriP-derived fragments (upper left), pG.β (lower left), pOS.E (upper right) and pOS.fE (lower right) are represented. Prom: promoter; polyA: SV40 polyA additional signal.

Fig. 2. The EBV-based AC achieves extremely efficient genetic transfection without drug selection. B16 cells were transfected by electroporation with pGEG.Luc (closed bars) or pG.Luc (hatched bars). The indicated days after the transfection, Luc activities were measured and standardized with protein amount. The mean ± SD of quadruplicate samples are shown. *P<0.05; **P<0.01

FIG. 3. Requirement of EBNA1, DS and FR for the high level transfection/expression. HeLa (A, left), CT26 (B, left), and 3T3 (C, left) cells as well as their transformants with EBNA1 gene (HLE, CT26E and 3T3E, respectively (A-C, right)) were co-transfected with pG.β and pGVP derivatives with the indicated fragments. Negative control groups were transfected with pG.β alone (no Luc). Indicated days later, cells were extracted and subjected for Luc and β-gal assays. The results are expressed as the mean ± SD of Luc/β-gal expression ratios of quadruplicate samples.

Fig. 4. The super-transfection of EBNA1 trans-activated preexisted Luc gene. HeLa/oriP-Luc (A), 3T3/oriP-Luc (B) and CT-16/oriP-Luc (C) cells were co-transfected with 0.45 pmol of the indicated plasmids and pG.β. Control cells were transfected with pG.β alone (-). Three days later, cells were harvested and subjected for Luc and β-gal assays. The results are shown as the mean ± SD of Luc/β-gal expression ratios of quadruplicate samples. *P<0.05; **P<0.01; N.S., Not significant.

Fig. 5. An oriP-bearing plasmid is transported into the nucleus in the presence of EBNA1. (A) K562 or KE

cells were treated with aphidocolin and 24 hs later transfected with pGVP-oriP. After 8 hs of culturing, DNA was differentially extracted from cytoplasm and nucleus and subjected to Southern blotting using oriP as probe. The arrow indicates the specific hybridization (2.5 kb). (B) KE cells were treated with aphidocolin, and 8 hs later cells were stained with PI and analyzed by FACS (lower panel). FACS profile of untreated KE cells are also shown (upper panel).

Fig. 6. EBNA1 accelerates cytoplasm-to-nuclear transport of oriP-bearing DNA in cells of human as well as mouse origins. HeLa (A, left), CT26 (B, left), and B16 (C, left) cells as well as their transformants with EBNA1 gene (HLE, CT26E and B16E, respectively (A-C, right)) were treated with aphidocolin to synchronize cell-cycle as in Fig. 6. Twenty-four hs later cells were transfected with pGVP-oriP. After 8 hs of culturing, DNA was differentially extracted from cytoplasm [C] and nuclear [N] fractions and subjected to real-time PCR using oriP-specific primers and probe. Means ± SD of relative copy numbers per cell are shown (n=3). *P<0.05; ***P<0.005; N.S., Not significant.

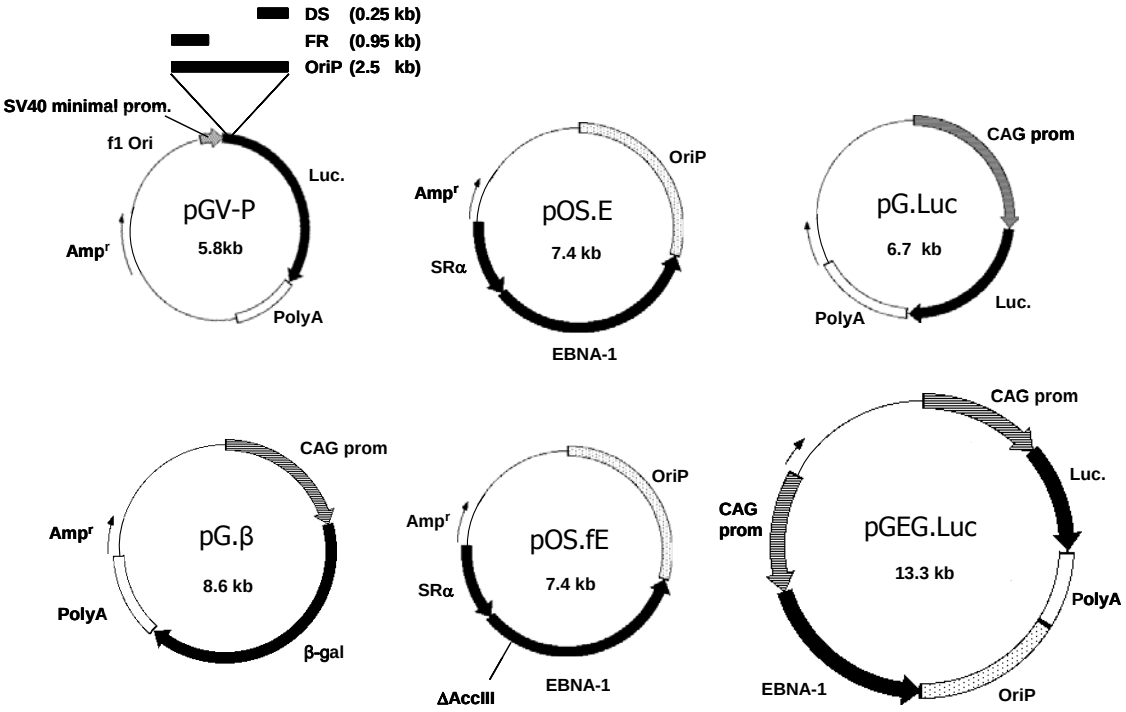


Fig. 1

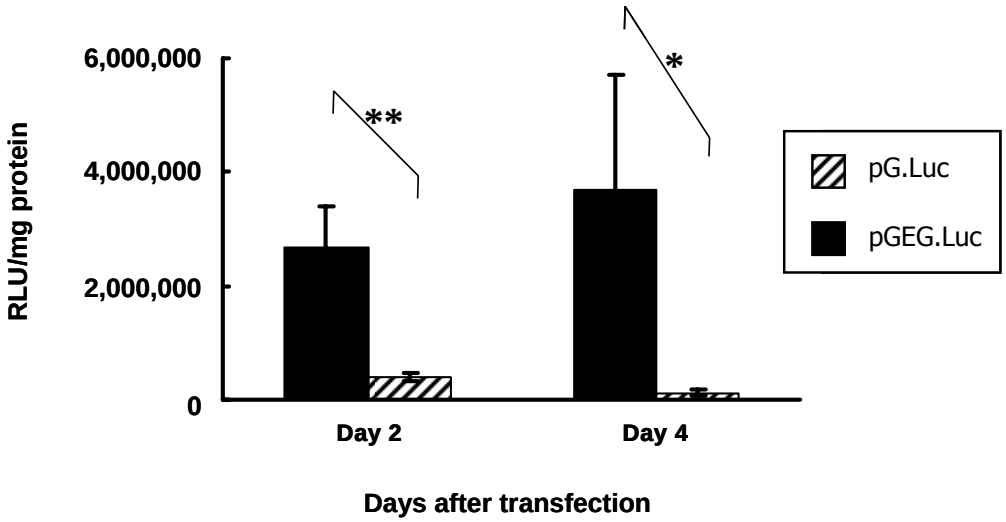
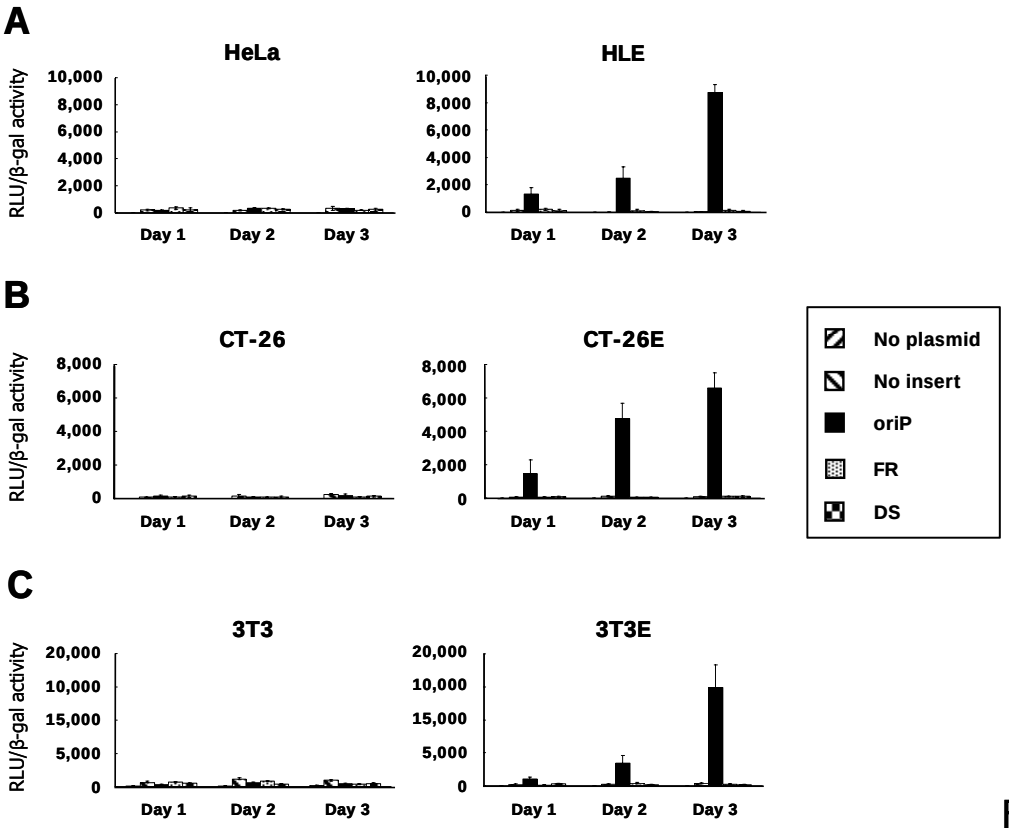


Fig. 2



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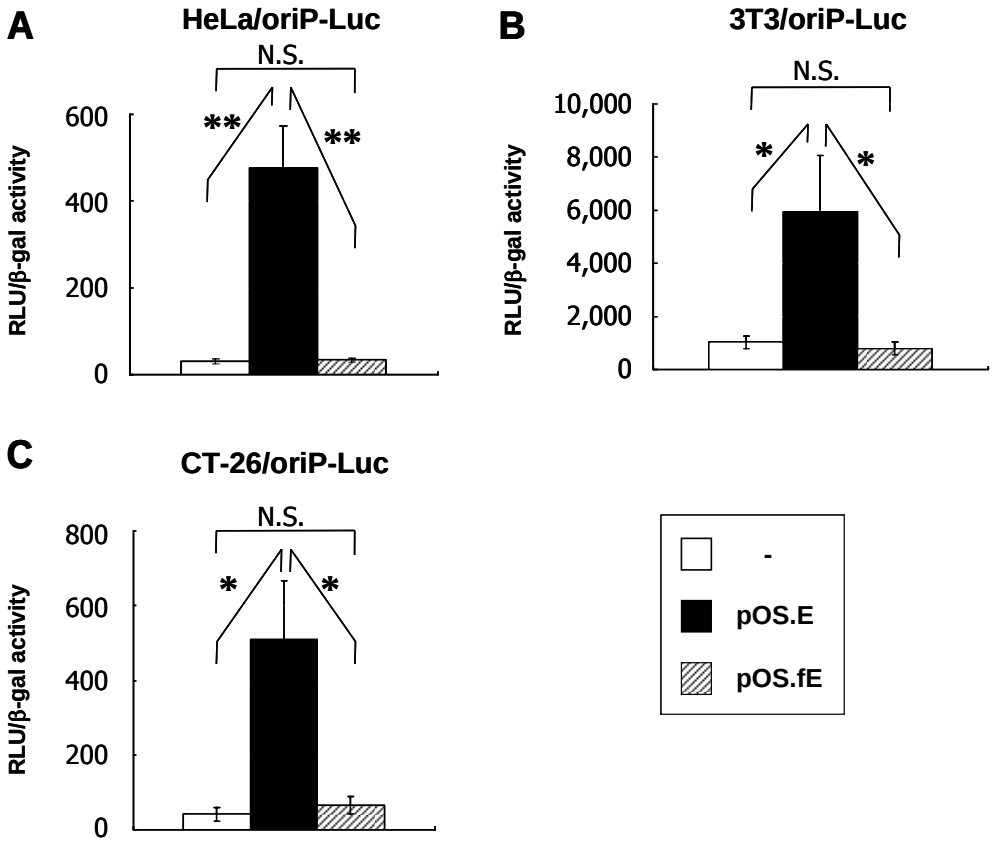

Fig. 4

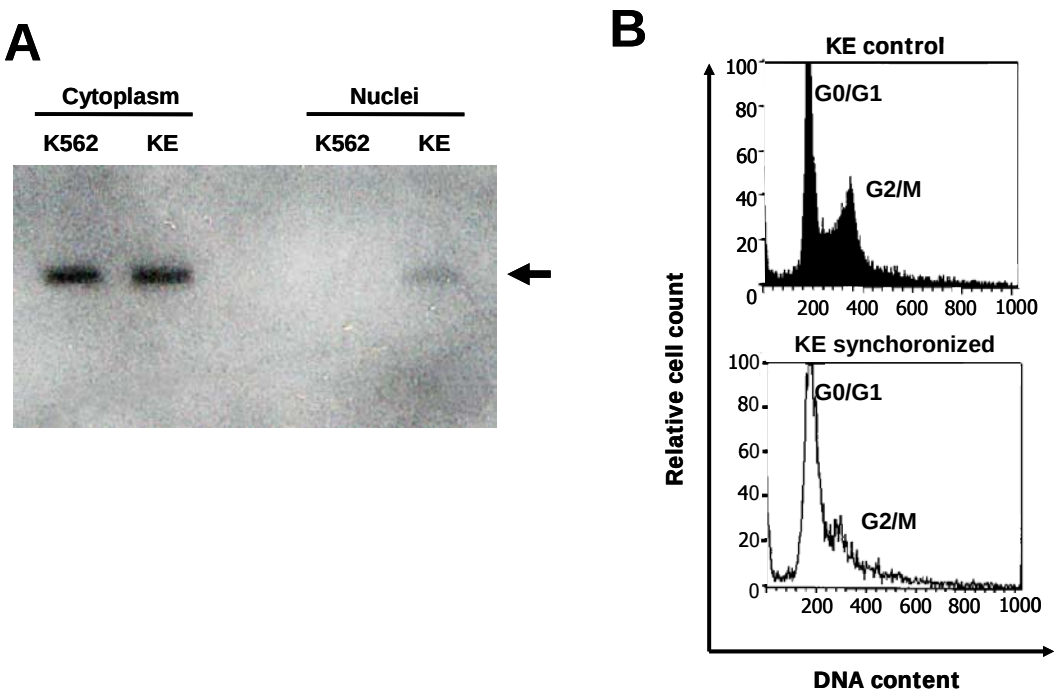

Fig. 5

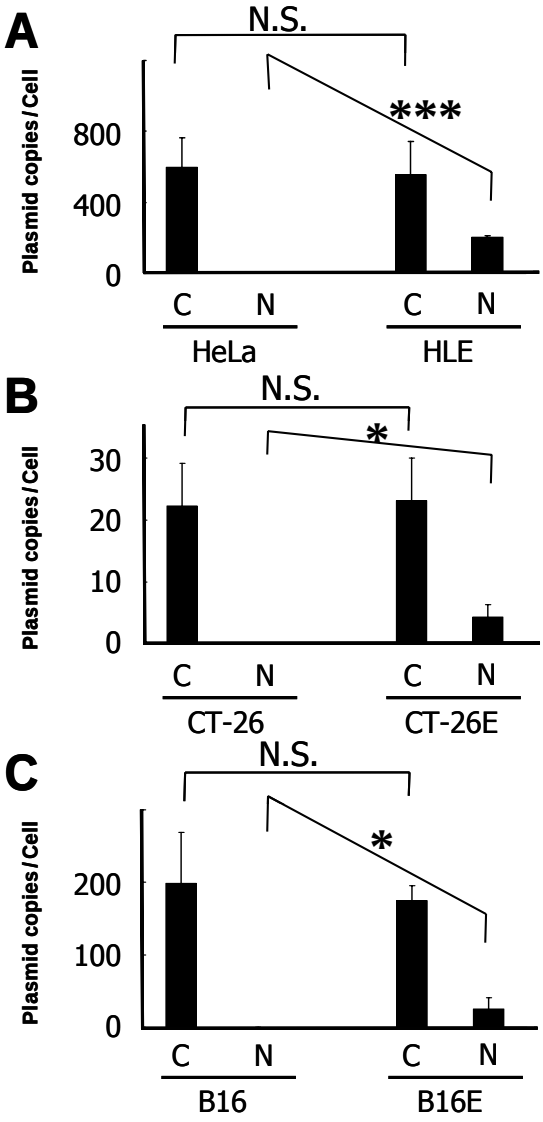

Fig. 6